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Title of Invention: Human serum albumin fragments

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Specification

1. Title of Invention:

Human serum albumin fragments

2. Claims:

1. Human serum albumin fragments, from the center part of human serum albumin.

2. A fragment in accordance with Claim 1 which has the amino acid sequence from the methionine in the 123rd position of human serum albumin to the proline in the 303rd position.

3. Fused proteins, consisting of central parts of human serum albumin and other polypeptides.

4. Fused proteins in accordance with Claim 1, consisting of signal peptide of coliform bacteria alkaline phosphatase and polypeptides which have the amino acid sequence from the methionine in the 123rd position of human serum albumin to the proline in the 303rd position.

5. Human serum albumin fragments, lacking the C terminus part of human serum albumin.

6. A fragment in accordance with Claim 5 which has the amino acid sequence from the aspartic acid in the 1st

position of human serum albumin to the proline in the 303rd position.

7. Fused proteins, consisting of fragments lacking the C terminus part of human serum albumin and other polypeptides.

8. Fused proteins in accordance with Claim 7, consisting of signal peptide of coliform bacteria alkaline phosphatase and polypeptides which have the amino acid sequence from the aspartic acid in the 1st position of human serum albumin to the proline in the 303rd position.

9. Human serum albumin fragments, lacking the N-terminus part of human serum albumin.

10. A fragment in accordance with Claim 9 which has the amino acid sequence from the methionine in the 123rd position of human serum albumin to the leucine in the 585th position.

11. Fused proteins, consisting of fragments lacking the N-terminus part of human serum albumin and other polypeptides.

12. Fused proteins in accordance with Claim 11, consisting of signal peptide of coliform bacteria alkaline phosphatase and polypeptides which have the amino acid sequence from the methionine in the 123rd position of human serum albumin to the leucine in the 585th position.

13. DNA sequences which encode the protein fragments mentioned in Claims 1, 5, or 9 or the fused proteins mentioned in Claims 3, 7, or 11.

14. Plasmids containing the DNA sequences mentioned in Claim 13.

15. Plasmids mentioned in Claim 14, which are expression plasmids that have control sequences for efficiently expressing the said DNA sequences in a host, upstream in the aforementioned DNA sequences.

16. Hosts, the characters of which have been transformed by the plasmids mentioned in Claim 14.

17. A method for manufacturing human serum albumin protein fragments or fused proteins containing the said fragments, characterized in that human serum albumin protein fragments or fused proteins containing the said fragments are expressed by culturing the hosts mentioned in Claim 16, and in the case in which fused proteins are expressed, the said human serum albumin protein fragments are cleaved from the said fused proteins as desired.

3. Detailed Explanation of Invention:

Field of Application in Industry

This invention concerns protein fragments consisting of parts of human serum albumin. These protein fragments are expected to have applications as carriers for transport and supply systems of drugs, etc.

Conventional Technology [Prior Art]

Human serum albumins are high-molecular-weight plasma proteins with a molecular weight of 66,458 which are synthesized in the human liver. In the body, they primarily have the important functions of regulating the osmotic pressure of the blood, bonding with various substances (e.g., fatty acids, metal ions such as Cu^{2+} and Ni^{2+}, bile bilirubin, many drugs, some water-soluble vitamins, etc.) and thus carrying them to target organs, supplying amino acids to tissues, etc. On the basis of these activities, human serum albumin is used in large quantities in the treatment of loss of albumin due to burns or gastritis, etc.; hypoalbuminemia, which occurs when albumin synthesis is reduced by cirrhosis of the liver; hemorrhagic shock; etc. Serum albumins also play the role of bonding nonspecifically with many drugs and transporting them in the blood. It is thought that drugs which bond with albumins move through the body due to blood circulation and are

eventually liberated from the albumins, pass through the capillary walls, and are dispersed, thus arriving at their sites of activity. Albumins have little toxicity and low antigenicity; they are easily decomposed in the body. They can be easily covalently bonded with drugs and formed into complexes. They have the advantages that they have excellent characteristics as substrates for drug delivery (drug carriers), and for many of them, bonding sites with various drugs have been determined or are suspected, so that they can be easily designed for the manufacturing of pharmaceutical preparations.

Fundamentally, almost all suspected bonding sites with many drugs are contained also in human serum albumin fragments, and are thought to be able to show activities as drug carriers. When used as carriers, etc., in transport and delivery systems for drugs, etc., from the point of view of limiting bonding ability with drugs, etc., it is predicted that it is more advantageous to use fragments of human serum albumin molecules, rather than the whole molecules.

In general, as methods for preparing fragments of proteins by cutting them, methods of using chemical substances such as cyanogen bromide or proteases such as trypsin, pepsin, etc. [to cut] proteins are known. However, in these methods, since the cutting sites are necessarily determined by the amino acid sequence of the proteins, it is not possible to cut them at any arbitrary desired site, and

therefore it is not possible to obtain ideal protein fragments. Therefore, such fragments cannot be obtained either from human serum albumin.

Problems Which This Invention Seeks to Solve

In contrast to this, by using recombinant DNA technology, it is possible to synthesize human serum albumin fragments consisting of any desired parts in suitable host cells. Therefore, this invention seeks to provide human serum albumin protein fragments by recombinant DNA technology, based on making DNA which encodes the desired protein fragments of human serum albumin, as well as a method for manufacturing them.

More specifically, this invention concerns human serum albumin fragments from the central part of human serum albumin and fused proteins composed of the said fragments and other polypeptides; human serum albumin fragments lacking the C-terminus parts of human serum albumin and fused proteins composed of the said fragments and other polypeptides, as well as human serum albumin fragments lacking the N-terminus parts of human serum albumin and fused proteins composed of the said fragments and other polypeptides; DNA which encodes these protein fragments or fused proteins; plasmids containing the said DNA; hosts whose characteristics have been transformed by the said plasmids; and a method for manufacturing human serum albumin protein fragments or fused proteins containing such

fragments which is characterized in that, by culturing the aforementioned hosts, human serum albumin protein fragments or fused proteins containing these fragments are expressed, and in case the fused protein fragments are expressed, the said human serum albumin protein fragments are cut from the said fused proteins as desired.

Concrete Explanation of Invention

The cDNA which encodes normal human serum albumin A has already been cloned (Public Patent Application No. 63-037453). Therefore, using this cDNA, it is possible to manufacture any desired fragments of normal human serum albumin A by genetic engineering methods.

This invention provides, as such fragments, (1) serum albumin fragments from the central parts of human serum albumin; (2) serum albumin fragments lacking the C-terminus of human serum albumin; and (3) serum albumin fragments lacking the N-terminus of human serum albumin. For example, this invention provides, as examples of albumin fragments from the central parts of human serum albumin, albumin fragments which contain the amino acid sequence from the methionine in the 123rd position of human serum albumin to the proline in the 303rd position; as examples of albumin fragments lacking the C-termini, albumin fragments which contain the amino acid sequence from the aspartic acid in the 1st position of human serum albumin to the proline in the 303rd position (these are sometimes called "mini-HSA");

and as examples of albumin fragments lacking the N-termini, albumin fragments which contain the amino acid sequence from the methionine in the 123rd position of human serum albumin to the leucine in the 585th position (these are sometimes called "contracted HSA").

These three types of albumin fragments of this invention have the following characteristics.

The albumin fragments of this invention all contain the central part of human serum albumin. This is because, up to now, 4 drug bonding sites have been discovered on the human serum albumin molecule which are contained within this central part (sites I-IV) [Sjöholm, I., Ekman, B. E., Kober, A., Ljugstedt-Pahlman, I., Seiving, B., and Sjödin, T., Mol. Pharmacol. 16, 767-(1979)]; at these sites, several amino acid residues which play important roles in bonding drugs are known [Fehske, K. et al., Biochem. Pharmacol. 30, 688-(1981)], and almost all of these are concentrated in the central part.

Sjöholm et al. have investigated the bonding sites of many kinds of drugs by using microcytes containing drugs uniformly dispersed in human serum albumins; they classify them as the diazepam site (site I), the digitoxin site (site II), and the warfarin site (site III). It also appears that, besides these, a tamoxifen site (site IV) or a bilirubin bonding site are present. Fehske et al. suspected that the amino acids which play important roles in the bonding sites

of diazepam, warfarin, and bilirubin are, respectively, Lys195 and His146, Arg145 and Trp214, and Lys199 and Lys240. On the other hand, the bonding sites for long-chain fatty acids such as palmitates appear to be in the C-terminus region [Reed, R. G., Feldhoff, R. C., Clute, O. L. and Peters, T., Tr. Biochemistry, 14, 4578- (1975); Berde, C. B., Hudson, B. S., Simoni, R. D. and Sklar, L. A., J. Biol. Chem., 254, 391- (1979)]; if the human serum albumin fragments from the central part of human serum albumin, or the human serum albumin fragments with the C-termini missing, of this invention are used, long-chain fatty acids cannot be bonded, and the production of drug carriers which can bond with diazepam, warfarin, etc., becomes possible.

Human serum albumins are high-molecular-weight proteins composed of 585 amino acids; they have 35 cysteine residues in their molecules, among which only the cysteine residue located closest to the N-terminus side (Cys-34) is present in a form which has a free SH group; the others form disulfide (S-S) bonds with each other; a total of 17 S-S bridges are formed in the molecule. It has recently been demonstrated that at least 2 enzymes [peptidylprolyl cis-trans isomerase and protein disulfide isomerase (PDI)] contribute to the process of forming higher-order (steric) structures of protein molecules; it is the latter, PDI, which plays an important role in forming S-S bridges. In the cells of mammals which produce serum albumin, PDI acts in

the processes of biosynthesis and intracellular transport of human albumin proteins, and the principal locations where PDI is known to be present are microsome fractions which contain microcytes. When human serum albumin is biosynthesized in prokaryotic cells, including coliform bacilli, the aforementioned reactions occur. There is no guarantee that correct S-S bridges will be formed in the molecules; the Cys-34 may cause a thiol/disulfide exchange reaction to occur with the cysteine residue in the molecule, producing a crossing of the S-S bridges and thus an isomer. Thus, when cysteine residues which have free SH groups are present, the efficiency with which proteins arise that take the normal steric form, which should be produced, is reduced, and the risk that proteins with abnormal steric structures will also be abnormal functionally becomes great. In contrast to this, in the albumin fragments of this invention, lacking the N-terminus part, which contain the amino acid sequence from the methionine in the 123rd position to the leucine in the 585th position, the Cys34 is removed, together with the other 6 cysteines located on the amino end side, and this risk is lessened.

In this invention, as typical examples of the 3 aforementioned types of albumin fragments, 3 kinds of albumin fragments with specific amino sequence ranges are mentioned; the 3 types of albumin fragments have the characteristics mentioned above, and all albumin fragments

which can exhibit these characteristics are included in the scope of this invention. For example, the range from the methionine in the 123rd position to the proline in the 303rd position was given as an example of the central part in which drug bonding sites are concentrated; the central part is not, however, limited to this range, but may be longer or shorter than the 123rd position to the 303rd position, as long as most of the drug bonding sites are included in it. Moreover, the range from the 304th position to the C-terminus was given as an example of the C-terminus region in which long-chain fatty acid bonding sites are present and which must therefore be removed, but it is not limited to this example; the range may be longer or shorter, as long as it contains the long-chain fatty acid bonding sites. Furthermore, the range from the N-terminus to the 122nd position is given as an example of the range of the N-terminus, which contains many cysteines and which therefore must be removed, but it is not limited to this range; it may be longer or shorter, as long as it is an N-terminus region which contains the cysteine in the 34th position.

Therefore, various albumin fragments can be designed, by referring to the following conditions, and fall within the scope of this invention. The essential condition for designing human serum albumin fragments is that fragments be selected which can be expected to retain steric structures required for bonding specific drugs. The points which need

to be noted are: (i) the S-S bridges present in the structures of natural human serum albumin must be kept in their original forms; (ii) therefore, an even number of cysteine residues must be present in the polypeptide chains forming the fragments; and (iii) cuts must not be made in the polypeptide chains which form loops by being bonded by S-S bridges. That is, several of the important domain structures, or at least the subdomain structures, which are present in natural human serum albumin molecules, must not be destroyed.

These points are especially important, for example, in cases in which one is trying to solubilize in vitro (in the test tube) human serum albumin fragments extracted from cells in their insolubilized forms and cause them to take the original normal steric forms (including also the S-S bonds). In such in vitro steric structure formation (refolding) reactions, it is possible to use peptidylprolyl cis-trans isomerase or PDI.

The method of making the cDNA which encodes the whole normal human serum albumin A or most of it is described concretely in Reference Example 1. The entire DNA which encodes the target protein fragment can be chemically synthesized by the usual methods, or it can be prepared from the aforementioned cDNA. When it is prepared from the cDNA, the cDNA which encodes all or most of the normal human serum albumin A is cut by a suitable restriction endonuclease

inside the 5′ end or 3′ end of the cDNA region which encodes the target protein fragment and the missing end code sequences are made up by chemically synthesized DNA. Otherwise, the cDNA can be cut by a suitable restriction endonuclease outside the 5′ end or 3′ end of the cDNA region which encodes the target protein fragment, and the excess DNA part is removed by an exonuclease. Of these two methods, different methods for processing the 5′ end and the 3′ end can be combined.

In the example of this invention, as the DNA which encodes the protein fragment composed of Met(123)-Pro(303) in the amino acid sequence of normal human serum albumin, synthetic DNA which encodes Met(123)-Ala(151) (Fig. 1) and cDNA which encodes Pro(152)-Pro(303) (the part shown in [ ] in Fig. 8-1 to Fig. 8-2), bonded together, are used. As the DNA which encodes a fused protein of the signal peptide of alkaline phosphatase and mini-HSA and which is used [in this invention], the DNA which encodes the signal peptide from alkaline phosphatase and human serum albumin A from Asp1 to Pro152, from the plasmid pUC-phoA-HSA-A, which contains the DNA which encodes the fused protein [composed of] the signal peptide of alkaline phosphatase and the whole length of the human serum albumin molecule, already described in Public Patent Application No. 63-037453, is fused with the DNA fragment which encodes Glu153-Pro303, cut from the plasmid pUC-HSA-I′, already described in Public

Patent Application Bulletin No. 63-268302. As the DNA which encodes the contracted HSA and is used [in this invention], the synthetic DNA part (Met123-Ala151) cut from the DNA which encodes the Met123-Pro303, made as described above, is bonded with the DNA sequence which includes the code region of Pro152-Leu585 and the 3' side non-selective region, cut from pUC-phoA-HSA-A, already described in Public Patent Application Bulletin No. 63-037453.

The DNA which encodes the normal human serum albumin fragments of this invention can be expressed in itself, but it can also be expressed in a form in which it is linked with DNA encoding other peptides, and a fused protein can be obtained. Various peptides can be used as fusion partners for obtaining this kind of fused protein. One of these, for example, is the signal peptide of coliform bacteria alkaline phosphatase. When the target human serum albumin fragment is obtained as this kind of fused protein, the signal peptide can be removed later and the human serum albumin fragment obtained.

In order to express a human serum albumin fragment, for example, the DNA which encodes the fused protein is inserted into a suitable expression vector, e.g., a plasmid, after which the said vector is introduced into the host, as described above. As the host for the expression, one can use eukaryotic cells such as animal cells or yeasts, or prokaryotic cells such as bacteria; the vector is chosen

according to the host. In expression plasmids in bacteria, the DNA which encodes the human serum albumin fragments or the fused proteins which include these fragments are placed at the base of the expression-controlling region, which includes a promoter and an SD sequence. For example, one can use trp promoter, lac promoter, lambda phage promoters (P_R, P_L), tufB promoter, or rrnB promoter, or hybrid promoters [composed] of these.

The transformation of the characteristics of the host, e.g., the coliform bacteria, by the expression vector, e.g., the plasmid, can be performed by the usual methods. The culturing of the coliform bacteria is performed by the usual methods. In order to produce the target proteins, after the coliform bacteria have multiplied to a specific level, the expression of the target genes is induced by performing an induction treatment. The method of the induction differs with the promoter being used; for example, when trp promoter is used, the induction can be performed by adding 3-β-indole acrylic acid to the culture medium.

In cases in which coliform bacteria are used as hosts, the target protein is accumulated primarily in the cells. Therefore, in order to recover the protein, the cultured bacteria are first collected and washed, if desired, after which they are resuspended in water or a buffer solution and the cells are destroyed. Since the target protein is contained primarily in the insoluble fraction, the insoluble

fraction is collected, by centrifugal separation, for example, and washed, if desired. Next, the insoluble fraction is transferred to a buffer solution for protein solubilization, e.g., a buffer solution containing sodium dodecyl sulfate and 2-mercaptoethanol, and the protein is solubilized.

Next, the said protein is recovered and purified according to the usual methods from this solution, which contains the fused protein of human serum albumin fragments. In order to obtain the target fused protein of human serum albumin fragments by splitting open the fused protein, the method of in vitro decomposition by means of coliform bacteria leader peptidase (signal peptidase I) [Zwizinski, C. and Wickner, W., J. Biol. Chem., 255, 7973 (1980)] can be used. Moreover, if cyanogen bromide is used on the fused protein, the fragment Cys124-Met298 is obtained.

Effectiveness of Invention

Since the albumin fragments which lack the C-terminus regions of this invention lack the long-chain fatty acid bonding sites which are present in the C-terminus , they have the advantage that they do not bond with long-chain fatty acids, but do bond with various drugs in their central regions. On the other hand, the albumin fragments which lack the N-terminus regions, lack Cys34 and many other cysteine residues and are advantageous for stable folding of proteins. Furthermore, the albumin fragments from only the

central part of the human serum albumin have the advantages of both of these.

Next, the manufacturing of the human serum albumin fragments of this invention will be explained concretely by means of actual examples.

In the actual examples, unless otherwise specifically mentioned, the enzyme reactions for treating the DNA were performed under the following conditions.

Restriction enzyme reactions

In the cases of Msp I (Nippon Gene Co., 10 units/μl), BamH I (Nippon Gene Co., 35 units/μl), Cla I (New England Biolabs, 5 units/μl), Hind III (Nippon Gene Co., 12 units/μl), and EcoR I (Nippon Gene Co., 12 units/μl): sterile distilled water was added to 1 μg DNA, 1 μl enzyme, and 3 μl 10X EcoR I buffer solution [1 M Tris•HcL (pH 7.5), 100 mM $MgCl_2$, 500 mM NaCl] to make 30 μl. The temperature was held at 37°C for 1 hour, to complete the cleavage. In the cases of Sal I and Xba I (Nippon Gene Co., 15 units/μl), in place of the 10X EcoR I buffer solution, 100 mM Tris-HCl (pH 7.5), 70 mM $MgCl_2$, 1.75 M NaCl, 70 mM 2-mercaptoethanol, 2 mM EDTA, and 0.1% bovine serum albumin were used.

In the cases of Pst I (Nippon Gene Co., 12 units/μl) and Sph I (Takara Shuzo Co., 10 units/μl), the concentration of the NaCl was doubled.

Treatment by bacterial alkaline phosphatase

Sterile distilled water was added to 1 μg DNA, 1 μl each restriction enzymes EcoR I and Hind III, and 2 μl 10 X EcoR I buffer solution to make 20 μl, and the temperature was held at 37°C for 1 hour, after which heating was performed at 90°C for 5 minutes to activate the enzymes. Next, 38 μl sterile distilled water and 2 μl bacterial alkaline phosphatase (Takara Shuzo Co., 0.5 units/μl) were added and the temperature was held at 37°C for 1 hour, after which phenol extraction was performed and the water layer obtained was used for ethanol precipitation.

T4 DNA ligase treatment

Sterile distilled water was added to, for example, 1 μg vector DNA, an equimolar quantity of DNA fragment to the vector DNA, 3 μl 10X ligase buffer solution [660 mM Tris-HCl (pH 7.5), 66 mM $MgCl_2$, 100 mM dithiothreitol, 1 mM ATP], and 1 μl T4 DNA ligase (Takara Shuzo Co., approximately 400 units/μl) to make 30 μl; the temperature was held at 16°C overnight.

5'-phosphorylation of synthetic fragments by T4 polynucleotide kinase

Quantities (approximately 30 pmoles) of the various DNA fragments in a solution (25 μl) containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 0.2 mM ATP are treated for 60 minutes at 37°C with 6 units of T4

polynucleotide kinase (Takara Shuzo Co.) to perform the 5'-phosphorylation. The solutions containing the phosphorylated fragments are mixed (total 100 μl) and kept for 5 minutes in a 100°C water bath, after which [this solution] is left to cool at room temperature; thus the annealing is performed. Two μl of T4 DNA ligase are added, and the temperature is kept at 16°C overnight, joining the fragments and making a double-chain fragment.

Coliform bacteria DNA polymerase I reaction

Sterile distilled water is added to 1 μg DNA, 1 μl DNA polymerase I (Klenow fragment, Takara Shuzo Co., 35 units/μl), 1 μl 1 mM dXTP (mixture of dATP, dGTP, dCTP, and TTP), and 3 μl 10X buffer solution [70 mM Tris-HCl (pH 7.5), 1 mM EDTA, 200 mM NaCl, and 70 mM $MgCl_2$] to make a total quantity of 30 μl; this was kept for 30 minutes at 37°C.

Actual Example 1. Synthesis of DNA encoding Met(123)-Ala(151)

The construction of a gene fragment which has a BamH I adhesion end on the 5' end, an Hpa II (Msp I) recognition sequence near the 3' end, and the double-chain part of which completely encodes the Met(123)-Ala(151) of human serum albumin was performed as follows. In order to express [these genes] efficiently in coliform bacteria, a sequence was designed which contained as many as possible of the codons

which are frequently used by genes which are expressed with high efficiencies in coliform bacteria (preferential codons). tRNA species with respect to these codons are generally present in large quantities in coliform bacteria [e.g., Ikemura, T. J., Mol. Biol., 151, 389-409 (1981); Gouy, M. and Gautier, C., Nucleic Acids Res., 10, 7055-7074 (1982)], and they can be expected to affect the translation efficiency.

In the actual synthesis, the following 4 oligonucleotides:

5'-GATCCATGTGCACCGCTTTCCACCACAACGAAGAAACCTTCC-3'

5'-AGGTATTTTTTCAGCAACGTTTCTTCGTTGTCGTGGAA
AGCGGTGCACATG-3'

5'-TGAAAAATACCTGTACGAAATCGCTCGTCGTCACCCG
TACTTCTACGCTCCGG-3'

5'-CGAAGAACAGCAGTTCCGGAGCGTAGAAGTACGGGTGA
CGACGAGCGATTTCGTAC-3'

were synthesized by using an automatic synthesizer (Applied Biosystems Model 380B), applying the phosphoamidite method developed by Caruthers et al. [Matteucci, M. D. and Caruthers, M. H., Tetrahedron Letters 21, 719 (1980)]. The DNA chains synthesized (approximately 30 pmoles) were treated in a solution of 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 0.2 mM ATP (50 μl), in the presence of 6 units of T4 polynucleotide kinase (Takara

Shuzo Co.), at 37°C, for 60 minutes, and their 5'-ends were phosphorylated.

The 4 phosphorylated fragments were mixed and kept in a 100°C water bath for 5 minutes, after which they were left to cool to room temperature, to perform the annealing. Two μl of T4 DNA ligase (800 units, Takara Shuzo Co.) were added and the temperature was held at 16°C overnight, joining the fragments and making a double-chain fragment. Next, this double-chain fragment was cut with Hpa II (Msp I) to obtain a 96 bp fragment.

Actual Example 2. Preparation of DNA fragment encoding human serum albumin fragment Met(123)-Pro(303) (Fig. 2)

The lambda gt11 human cDNA clone (HSA-1A) lacking the part which encodes the amine end side of normal human serum albumin and containing a sequence in which the codon coding the 304th serine is changed to a translation termination codon (Reference Example 1, Fig. 6) was cut by EcoR I and the human serum albumin cDNA part was taken out; this was inserted into the EcoR I site of plasmid pUC19, making plasmid pUC-HSA-I.

pUC-HSA-I was cut with Pst I and the 5'-end phosphoric acid group produced was removed by treating with bacterial alkaline phosphatase; after this, the result was cut with Hpa II (Msp I), and the 750 bp fragment was removed. This 750 bp fragment was joined with the 96 bp fragment

synthesized in Actual Example 1 by means of T4 DNA ligase, using the correspondence of the cohesive ends of Hpa II (Msp I). After this, it was joined with the larger fragment of the double digestion product of BamH I and Pst I of pUC19, by means of T4 DNA ligase, and the pSAL II plasmid was obtained.

Actual Example 3. Preparation of fused protein expression plasmid pAT-trp-phoA-SAL II (Fig. 3)

pSAL II was treated with BamH I to perform ring opening; the ends were treated with coliform bacteria DNA polymerase I to make smooth ends, after which it was cut with Hind III to obtain a 750 bp fragment containing HSA cDNA. On the other hand, plasmid pUC-phoA (Reference Example 2), [made by] incorporating an artificial leader sequence encoding the signal peptide of coliform bacteria phosphatase (phoA) into the pUC 19 plasmid, was cut with Hpa II (Msp I), and after the ends were smoothed with coliform bacteria DNA polymerase I, it was cut with EcoR I to obtain a 69 bp fragment containing the leader sequence. This fragment and a 750 bp fragment containing part of the normal human serum albumin cDNA derived from pSAL II were joined with T4 DNA ligase, and this was further joined with the larger of the fragments among the double digestion products of EcoR I and Hind III of pUC 19; thus, the pUC-phoA-SAL II plasmid, in which the leader sequence and the HSA cDNA part are joined, was obtained. Between the leader sequence encoding the phoA

signal peptide and the part of the HSA cDNA joined in this way, the nucleotide sequence GGATCC was produced, as an adaptor sequence, and since the two amino acids Gly-Ser are encoded, the fused protein actually synthesized by these fused genes takes the structure of the phoA signal peptide - Gly-Ser-Met123 to pro 303.

In order to express the fused protein in coliform bacteria, the pAT-trp-phoA-HSA-A (Reference Examples 3 and 4; Public Patent Application Bulletin No. 63-037453), which was used in the expression of the fused protein of phoA signal peptide-normal human serum albumin, was used. The pAT-trp-phoA-HSA-A was doubly digested by EcoR I and Hind III, and the larger of the fragments, which did not contain the phoA leader sequence-HSA cDNA part, was joined with the 800 bp fragment obtained by the double digestion of the pUC-phoA-SAL II plasmid by EcoR I and Hind III, by means of T4 DNA ligase, and the pAT-trp-phoA-SAL II plasmid was obtained.

By introducing the pAT-trp-phoA-SAL II plasmid into the coliform bacterium HB101, using the character transformation method, the coliform bacterium HB101 (pAT-trp-phoA-SAL II) was obtained.

This coliform bacterium was entrusted to the Microbiology Industry Technology Institute of the Agency of Industrial Science and Technology, as Bikokenkinki No. 10308 (FERM P-10308).

Actual Example 4. Expression of fused protein

The fused protein of coliform bacteria alkaline phosphatase signal peptide and human serum albumin fragments, incorporating the pAT-trp-phoA-SAL II, was expressed as follows.

Culturing

A coliform bacteria strain HB101 which had pAT-Trp-phoA-SAL II was cultured in 5 ml of Luria (LB) medium (1% Bactotryptone, 0.5 % yeast extract, 0.5% NaCl) containing 25 μg/ml ampicillin, and culturing with agitation was performed at 37°C for 18 hours. 0.2 ml of this culture liquid was inoculated into 5 ml of M9-CA medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.5% NaCl, 0.1% NH_4Cl, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, 0.8% casamino acid) containing 25 μg/ml ampicillin, and culturing was performed for 30 minutes at 37°C. After this, the inducing substance 3-β-indole acrylic acid (IAA) was added to make 20 μg/ml. Culturing with agitation was performed thereafter for 5-7 hours at 37°C.

Extraction of insoluble fraction

The culture liquid which had been cultured in the above-described manner was centrifuged at 7000 rpm for 5 minutes to collect the bacteria. The precipitated bacteria were resuspended in 20% sucrose, 25 mM Tris-HCl (pH 7.5), 10

mM EDTA, and 1 mM PMSF (phenylmethylsulfonyl fluoride), and 0.2 mg/ml egg white lysozyme was added. The outer membranes were consumed by letting this stand at 37°C for 15 minutes, and the protoblasts (spheroblasts) were obtained. This suspension was transferred onto ice and cooled, after which it was centrifuged at 10,000 rpm for 10 minutes and the spheroblasts were precipitated. These spheroblasts were resuspended in a 20% sucrose solution [25 mM Tris-HCl (pH 7.5), 10 mM EDTA], and then pulverized in an ice bath by means of a Polytron [phonetic; poritoron] homogenizer (dial value: 8). The pulverized solution was centrifuged at 15,000 rpm for 20 minutes, at 4°C, and a bacteria residue was obtained. This bacteria residue was resuspended in 25 mM Tris-HCl (pH 7.5), and the suspension was centrifuged at 15,000 rpm for 20 minutes, at 4°C. This operation was performed once more, and the precipitate obtained was used as the insoluble fraction.

SDS-polyacrylamide gel electrophoresis

1) Analysis of bacteria total protein

0.5 ml of culture solution was centrifuged at 7000 rpm for 5 minutes, and the bacteria were collected. The bacteria were floated in 10 μl SDS-sample solution [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% sucrose, 5% 2-mercaptoethanol] and treated at 100°C for 5 minutes. The result was applied to an SDS-polyacrylamide gel [Laemmli's method: Nature (London)

277, 680 (1970)], with a separation gel concentration of 10%, and electrophoresis was performed.

2) Analysis of insoluble fraction

The residue was resuspended in 25 mM Tris-HCl (pH 7.5); part of this was taken and diluted with the SDS-sample solution. The insoluble protein was solubilized by treating at 100°C for 5 minute, and gel electrophoresis was performed.

3) Staining and destaining

After the electrophoresis was completed, the gel was immersed for 30 minutes to 1 hour in a staining solution (0.25% Coomasie Brilliant Blue, 45% ethanol, 10% acetic acid) and stained. The stained gel was transferred to a destaining apparatus (Bio-rad Co., Model 556) filled with destaining solution (5% methanol, 10% acetic acid) and destained.

Western blot and immune cross reactions

After the SDS-polyacrylamide gel electrophoresis was completed, the gel was removed from the glass plate. A nitrocellulose filter (Bio-Rad Co., Trans-blot$^{(R)}$) cut to the gel size and 3 MM filter paper made by the Whatman Co. (2 sheets) were immersed in a blotting solution (0.3% Tris, 1.44% glycine, 20% methanol). One filter paper sheet, the gel, the filter, and [the other] filter paper sheet were piled, in that order, on a Scotch pad previously soaked in

(Bio-rad Co.), and 16.7% methanol, and reaction was performed for 15 minutes. Next, the filter was left standing in water for 30 minutes. The material which cross-reacted with the anti-human albumin antibodies was stained a deep violet at a certain place (Fig. 4). The expressed product of this invention was observed at the position of molecular weight 21,000.

Actual Example 5. Preparation of plasmid pUC-phoA-mHSA, containing a DNA sequence encoding a fused protein of coliform bacteria alkaline phosphatase signal peptide and mini-HSA (Fig. 9)

The pUC-phoA-HSA-A mentioned in Reference Example 3, containing a DNA sequence encoding a fused protein of coliform bacteria alkaline phosphatase signal peptide and mature human serum albumin A, was doubly digested with EcoR I and Msp I; the region from immediately before the methionine codon of the amine end of the signal peptide of the alkaline phosphatase to the codon of the 152nd position proline of the mature human serum albumin A (approximately 500 bp) was cut out. On the other hand, the recombinant plasmid pUC-HSA-I', containing a DNA sequence in which, of the precursor prepro human serum albumin A, the mature human serum albumin A was encoded up to the proline of the 303rd position, but the codon of the 304th position serine (TCA) was replaced with an opal codon (TGA), was doubly digested with Msp I and Xba I; a DNA fragment of approximately 610

bp, encoding from the 153rd position glutamic acid to the 356th position threonine (however, since the translation stops with the 304th position opal codon, in reality the region up to the 303rd position proline was encoded), was obtained. These 2 DNA fragments were joined with the larger of the products of the double digestion of the plasmid vector pUC18 with EcoR I and Xba I (approximately 2660 bp), forming the recombinant plasmid pUC-phoA-mHSA, containing a DNA sequence encoding a fused protein (phoA-mHSA) composed of the signal peptide of coliform bacteria alkaline phosphatase and the region Asp1-Pro303 of mature human serum albumin A.

Actual Example 6. Preparation of plasmid pAT-trp-phoA-mHSA, containing a DNA sequence for expressing the fused protein phoA-mHSA, composed of coliform bacteria alkaline phosphatase signal peptide and mini-HSA (Fig. 9)

The aforementioned plasmid pUC-phoA-mHSA was doubly digested with EcoR I and Hind III, and the DNA sequence encoding the fused protein of coliform bacteria alkaline phosphatase signal peptide and mini-HSA was cut out. This was joined with the larger DNA fragment cut by double digestion with EcoR I and Hind III from the recombinant plasmid pAT-trp-phoA-HSA-A, used in the preparation of the fused protein of coliform bacteria alkaline phosphatase signal peptide and mature human serum albumin A. The recombinant plasmid pAT-trp-phoA-HSA-A has a structure in

which a DNA sequence encoding coliform bacteria alkaline phosphatase signal peptide and mature human serum albumin A and its 3' side non-translation sequence are placed downstream from the EcoR I recognition site, which is downstream from the coliform bacteria tryptophan promoter, and the Hind III recognition site is located at the very end. Therefore, the larger of the DNA fragments obtained by double digestion using EcoR I and Hind III takes a form in which the DNA sequence encoding coliform bacteria alkaline phosphatase signal peptide and mature human serum albumin A is lacking; by joining this with the DNA sequence encoding the fused protein of coliform bacteria alkaline phosphatase signal peptide and mini-hSA, it was possible to construct the recombinant plasmid pAT-trp-phoA-mHSA, which has a structure in which the said fused protein could be expressed under the control of the coliform bacteria tryptophan promoter.

The pAT-trp-phoA-mHSA plasmid was introduced by the characteristic transformation method into coliform bacterium HB101 and coliform bacterium HB101 (pAT-trp-phoA-mHSA) was obtained. This bacterium was entrusted to the Microbiology Industry Technology Institute of the Agency of Industrial Science and Technology, as Bikokenkinki No. 10952 (FERM P-10952).

Actual Example 7. Preparation of recombinant plasmid pUC-tHSA, containing the DNA encoding contracted HSA

The aforementioned recombinant plasmid pSAL II contains a DNA sequence which can encode [the region] of mature human serum albumin A from Met123 to Pro303; the DNA fragment encoding Met123-Ala151 (approximately 90 bp) was cut from it by double digestion with BamH I and Msp I. On the other hand, a double digestion of the aforementioned plasmid pUC-phoA-HSA-A was performed with Msp I and Hind III, and a fragment of approximately 1350 bp, encoding [the region] from Pro152 [to] Leu585, the carboxyl end of mature human serum albumin A, and containing its 3' side non-translation sequence, was obtained. These 2 fragments were joined with a DNA fragment of approximately 2660 bp, obtained by the double digestion of pUC18 with BamH I and Hind III, and a recombinant plasmid pUC-tHSA, containing a DNA sequence encoding Met123-Leu585 (contracted HSA), was constructed.

Actual Example 8. Preparation of recombinant plasmid pAT-trp-tHSA, for expressing contracted HSA (Fig. 10)

In order to express the contracted HSA (Met123-Leu585) directly, and not in the fused form, a coliform bacteria tryptophan promoter was used. Using the plasmid vector pAT153 as a foundation, the plasmid vector for expression pAT-trp, incorporating the promoter from the coliform bacteria tryptophan operon and the SD sequence of trpL, was cut at the Cla I recognition site, which is downstream from the sequence derived from the tryptophan operon, and ring-opening was performed. After this, a treatment was performed

with coliform bacteria DNA polymerase I and the single-chain part of the end was buried by a nucleotide polymerization reaction. Next, cutting was performed with Sph I, and the larger of the DNA fragments was obtained. On the other hand, the recombinant plasmid pSAL II, containing a DNA sequence encoding the Met123-Pro303 (SAL II) of mature human serum albumin A, was cut at the BamH I recognition site, immediately before the Met123 codon, after which a nucleotide polymerization reaction was performed by using coliform bacteria DNA polymerase I, and the single-chain part of the end was buried. Next, cutting was performed with Sph I and the smaller of the DNA fragments, containing a DNA sequence encoding SAL II, was obtained. These 2 DNA fragments were joined to prepare a recombinant plasmid pAT-trp-SAL II, in which a DNA sequence encoding SAL II was placed downstream from the sequence derived from the coliform bacteria tryptophan operon. After this pAT-trp-SAL II was cut at the Sal I recognition site, located downstream from the SAL II DNA sequence, the single-chain DNA part was buried with coliform bacteria DNA polymerase I, and it was cut again at the site of the 5′ end of the SAL II DNA by means of BamH I, cutting and removing the SAL II DNA. The larger of the DNA fragments obtained in this way was joined with a DNA fragment containing a DNA sequence encoding contracted HSA, obtained by cutting the pUC-tHSA plasmid with Hind III, burying the single-chain part with coliform bacteria DNA polymerase I, and cutting with BamH I; in this

way, the recombinant plasmid pAT-trp-tHSA for expressing contracted HSA was constructed. The pAT-trp-tHSA plasmid was introduced into coliform bacterium HB101 by the characteristic transformation method, and coliform bacterium HB101 (pAT-trp-tHSA) was obtained. This bacterium was entrusted to the Microbiology Industry Technology Institute of the Agency of Industrial Science and Technology, as Bikokenkinki No. 10950 (FERM P-10950).

Actual Example 9. Preparation of recombinant plasmid pAT-trp-phoA-tHSA, which expresses the fused protein phoA-tHSA, composed of coliform bacteria alkaline phosphatase signal peptide and contracted HSA (Fig. 11)

The recombinant plasmid pAT-trp-phoA-SAL II for expressing the fused protein of coliform bacteria alkaline phosphatase signal peptide and SAL II was cut at the SAL I recognition site, downstream from the SAL II DNA sequence, and ring opening was performed, after which the end was treated with coliform bacteria DNA polymerase I and the single-chain part was buried. Next, it was cut at the BamH I recognition site which is in the spacer region between the DNA sequences coding the alkaline phosphatase signal peptide and SAL II, and a DNA fragment was obtained which contained a structure in which a DNA sequence encoding the alkaline phosphatase signal peptide was connected downstream from the DNA sequence derived from the tryptophan operon. On the other hand, after the pAT-trp-tHSA was cut with Hind III, a

treatment with DNA polymerase I was performed, burying the single-chain part, and a DNA sequence encoding contracted HSA was cut out by cutting with BamH I. These 2 DNA fragments were connected and a recombinant plasmid pAT-trp-phoA-tHSA, which expresses the fused protein phoA-tHSA in a form in which the alkaline phosphatase signal peptide and contracted HSA are sandwiched by spacers composed of the dipeptide Gly-Ser encoded by the BamH I recognition sequence GGATTCC, was constructed. The pAT-trp-phoA-tHSA plasmid was introduced into the coliform bacterium HB101 by means of the characteristic transformation method, and the coliform bacterium HB101 (pAT-trp-phoA-tHSA) was obtained. This bacterium was entrusted to the Microbiology Industry Technology Institute of the Agency of Industrial Science and Technology, as Bikokenkinki No. 10951 (FERM P-1051 [sic]).

Actual Example 10. Expression of fused proteins composed of alkaline phosphatase signal peptide and mini-HSA or contracted HSA and the single contracted HSA molecule

The fused proteins of coliform bacteria alkaline phosphatase signal peptide and human serum albumin fragments or contracted human serum albumin A alone, were expressed by means of pAT-trp-phoA-mHSA, pAT-trp-tHSA, or pAT-trp-phoA-tHSA as follows.

Culturing

Coliform bacteria strains HB101 which had pAT-trp-phoA-mHSA, pAT-trp-tHSA, or pAT-trp-phoA-tHSA were cultured in 5

ml of Luria (LB) medium (1% Bactotryptone, 0.5 % yeast extract, 0.5% NaCl) containing 25 μg/ml ampicillin, and culturing with agitation was performed at 37°C for 18 hours. 0.2 ml of this culture liquid was inoculated into 5 ml of M9-CA medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.5% NaCl, 0.1% NH_4Cl, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, 0.8% casamino acid) containing 25 μg/ml ampicillin, and culturing was performed for 30 minutes at 37°C. After this, the inducing substance 3-β-indole acrylic acid (IAA) was added to make 20 μg/ml. Culturing with agitation was performed thereafter for 5-7 hours at 37°C.

Extraction of insoluble fraction

The culture liquid which had been cultured in the above-described manner was centrifuged at 7000 rpm for 5 minutes to collect the bacteria. The precipitated bacteria were resuspended in 20% sucrose, 25 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 1 mM PMSF (phenylmethylsulfonyl fluoride), and 0.2 mg/ml egg white lysozyme was added. The outer membranes were consumed by letting this stand at 37°C for 15 minutes, and the protoblasts (spheroblasts) were obtained. This suspension was transferred onto ice and cooled, after which it was centrifuged at 10,000 rpm for 10 minutes and the spheroblasts were precipitated. These spheroblasts were resuspended in a 20% sucrose solution [25 mM Tris-HCl (pH 7.5), 10 mM EDTA], and then pulverized in an ice bath by means of a Polytron homogenizer (dial value: 8). The

pulverized solution was centrifuged at 15,000 rpm for 20 minutes, at 4°C, and a bacteria residue was obtained. This bacteria residue was resuspended in 25 mM Tris-HCl (pH 7.5), and the suspension was centrifuged at 15,000 rpm for 20 minutes, at 4°C. This operation was performed once more, and the precipitate obtained was used as the insoluble fraction.

SDS-polyacrylamide gel electrophoresis

1) Analysis of bacteria total protein

0.5 ml culture solution was centrifuged at 7000 rpm for 5 minutes, and the bacteria were collected. The bacteria were floated in 10 μl SDS-sample solution [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% sucrose, 5% 2-mercaptoethanol] and treated at 100°C for 5 minutes. The result was applied to an SDS-polyacrylamide gel [Laemmli's method: Nature (London) 277, 680 (1970)], with a separation gel concentration of 10%, and electrophoresis was performed.

2) Analysis of insoluble fraction

The residue was resuspended in 25 mM Tris-HCl (pH 7.5); part of this was taken and diluted with the SDS-sample solution. The insoluble protein was solubilized by treating at 100°C for 5 minutes, and gel electrophoresis was performed.

3) Staining and destaining

After the electrophoresis was completed, the gel was immersed for 30 minutes to 1 hour in a staining solution

(0.25% Coomasie Brilliant Blue, 45% ethanol, 10% acetic acid) and stained. The stained gel was transferred to a destaining apparatus (Bio-rad Co., Model 556) filled with destaining solution (5% methanol, 10% acetic acid) and destained.

Western blot and immune cross reactions

After the SDS-polyacrylamide gel electrophoresis was completed, the gel was removed from the glass plate. A nitrocellulose filter (Bio-Rad Co., Trans-blot(R)) cut to the gel size and 3 MM filter paper made by the Whatman Co. (2 sheets) were immersed in a blotting solution (0.3% Tris, 1.44% glycine, 20% methanol). One filter paper sheet, the gel, the filter, and [the other] filter paper sheet were piled, in that order, on a Scotch pad previously soaked in the blotting solution, and another Scotch pad was placed on top; this was set in a blotting apparatus (Tefco Co., Model TC-808). The apparatus was filled with blotting solution and electrophoresis was performed at 200 mA for 1 hour.

After the electrophoresis was completed, the filter was removed from the gel and a treatment was performed for 10 minutes with TBS solution [25 mM Tris-HCl (pH 7.5), 0.5 M NaCl]. After a treatment was performed for 30 minutes with TBS solution containing 3% gelatin, the filter was

transferred to TBS solution containing 0.025% Tween-20 (abbreviated below as "TTBS solution"), and a treatment was performed for 5 minutes, after which the same operations were repeated. The IgG fraction of anti-human albumin rabbit serum (Cappel Co.) was diluted 2000-fold with TTBS solution containing 1% gelatin, the filter was transferred to this solution, and a treatment was performed for 2-18 hours. Next, the filter was transferred to TTBS solution and treated for 5 minutes. This operation was repeated 2 more times. The filter was transferred to a solution of goat anti-rabbit IgG antibodies conjugated to horseradish-peroxidase (Bio-rad Co.), diluted 3000-fold with TTBS solution containing 1% gelatin, and a treatment was performed for 2 hours. After this treatment, the filter was washed twice with TTBS solution and once with TBS solution (5 minutes each time). The filter was transferred to a TBS solution containing 0.015% H_2O_2, 0.05% HRP chromogen reagent (Bio-rad Co.), and 16.7% methanol, and reaction was performed for 15 minutes. Next, the filter was left standing in water for 30 minutes. The material which cross-reacted with the anti-human albumin antibodies was stained a deep violet at certain places (Fig. 12). The expressed products of cross reactions of phoA-mHSA, contracted HSA, and phoA-tHSA with the corresponding anti-human serum albumin antibodies were observed at the positions of approximate molecular weights 37,000, 49,000, and 51,000, respectively.

Reference Example 1. Screening of clones containing normal human serum albumin A cDNA

For the sake of screening clones containing normal human serum albumin A cDNA by plaque hybridization, a human liver cDNA library made by using lambda gt11 of the U.S. Clontech Co. as the vector was used. The lambda gt11 recombinant phages were inoculated, using coliform bacterium Y1090 as the host, and a total of 5.5×10^5 character-transformed plaques were formed on an LB agar-agar medium (Luria medium + 1.5% agar-agar). After the recombinant DNA was transferred to a membrane filter (Amersham Co. Hybond-N), the screening was performed by using 3 kinds of synthetic oligonucleotides labelled with ^{32}P radioactive isotope (specific activities $\geq 10^7$cpm/μg) as probes [Benton and Davis, Science 196, 180-182 (1977)]. These 3 probes are the same sequences, respectively, as, among the human serum albumin cDNA sequences reported by Lawn et al. [Nucleic Acids Res, 9, 6103-6114 (1981)], the one containing the 5' non-translation region (the part from 12 nucleotides upstream from the ATG codon of the translation start to the nucleotide before the ATG codon) and the translation region (the methionine codon of the amine end, i.e., the part encoding the 9th amino acid leucine from the ATG) (HSA-1); the one encoding the 260th leucine from the 248th glycine (HSA-2); and the one containing the part which encodes the carboxyl end 585th leucine from the 576th valine and the 3'

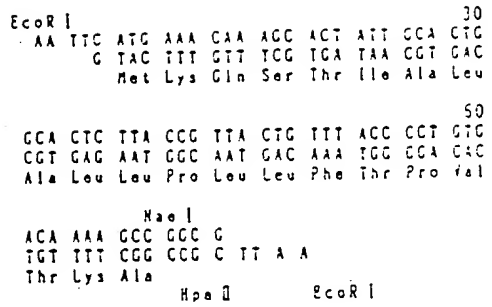
non-translation region composed of the following 6 nucleotides (HSA-3). The base sequences of these probes are shown in Fig. 5. The synthesis of these probes was performed by using an automatic DNA synthesizer; the labelling was performed by using [γ-^{32}P] ATP and polynucleotide kinase. Among the 200 lambda gt11 clones which gave positive signals with HSA-2, DNA was prepared from 4 clones [Blattner et al., Science 202, 1279-1284 (1978)]; this was digested with EcoR I enzyme, and the Southern blot of the digested material was hybridized with the HSA-2 probe [Southern, E., J. Mol. Biol. 503-517 (1975)]. The hybridized fragments were obtained from 3 clones; their lengths were 1.8 kb, 1.4 kb, and 1.3 kb. Among these, the fragments with the lengths of 1.8 kb and 1.3 kb were sub-cloned with the pUC19 vector. These subclones were screened by colony hybridization [Grunstein and Hogness, Proc. Natl. Acad. Sci. USA 72, 3961-3965 (1975)], using HSA-1 and HSA-3, respectively, as probes. As a result, a clone lambda gt11 (HSA I-A) which hybridized only with HSA-3 was obtained. Various DNA fragments of this clone were transferred to the vectors for determining base sequences M13mp18 and mp19 RF-DNA, and the base sequences were determined by the stain deoxynucleotide termination method [Sanger, F., Nicklen, S., and Coulson, A. R., Proc Natl. Acad. Sci. USA 74, 5463-5467 (1977)]. On the other hand, with 20 of the clones which gave positive signals in the plaque hybridization of the lambda gt11 clones performed

by using HSA-2 as the probe, plaque hybridization was performed again using HSA-1 as the probe, and one clone lambda gt11 (HSA-II) which gave a positive signal was obtained. Phage DNA was prepared from this, and the EcoR I-digested material was Southern-hybridized using HSA-1 as the probe; the 1.25 kb fragment (HSA-II) was confirmed to hybridize with the probe. The base sequence of this fragment was determined by the stain deoxynucleotide termination method. HSA-II did not hybridize with the HSA-3 probe. As a result, it was found that HSA-II lacked the part which encodes the carboxyl end side, HSA-I-A lacks the part which encodes the amine end side of human serum albumin, and the codon which encodes the 304th serine (TCA) was changed to the opal codon TGA of the translation termination codon. Fig. 6 shows the limiting enzyme maps of these two DNA fragments. The accurate positions of the amino acid recognition sites were obtained from the final base sequence.

Reference Example 2. Preparation of plasmid pUC-phoA

The plasmid pUC-phoA, containing chemically-synthesized DNA which encodes the signal peptide of coliform bacteria alkaline phosphatase, was prepared in the following manner.

A DNA fragment containing the following base sequence, which encodes the signal peptide of coliform bacteria alkaline phosphatase, was constructed from chemically-synthesized fragments.



The EcoR I recognition sequences at both ends were prepared in order to perform an insertion into the EcoR I site of the PUC plasmid; the Hpa II recognition sequence was prepared in order to fuse the HSA-A mature gene afterward; and the Nae I recognition sequence was prepared so that [the DNA fragment] would be cut directly after the codon encoding the last amino acid (21st alanine) constituting the signal peptide and leave smooth ends, and this could be fused directly with the DNA sequence encoding the mature protein. Two DNA chains composed of 72 nucleotides were synthesized by using an automatic DNA synthesizer (Applied Biosystems Model 380B), applying the phosphoamidite method described in Matteucci, M. D. and Caruthers, M. H., Tetrahedron Letters 21, 719 (1980). Quantities (21 pmoles) of each of the synthesized DNA chains were treated at 37°C for 60 minutes in, e.g., solutions (50 μl) containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 0.2 mM ATP, in the presence of 6 units of T4 polynucleotide kinase (Takara Shuzo Co.), to perform phosphorylation of the 5′ ends.

The solutions containing the aforementioned 2 phosphorylated DNA chains were mixed (total 100 μl) and put into a 100°C water bath; next, the resulting material was allowed to cool to room temperature, so that annealing was performed. To increase the probability that, when the annealed double-chain phosphorylated DNA was incorporated into the pUC 19 plasmid, a recombinant plasmid would be obtained in which the said DNA would be incorporated, the pUC 19 plasmid which was the vector was cut with EcoR I, after which the phosphate group of the 5' end was removed; in this way, the probability that relinking would occur by the DNA ligase treatment could be reduced to a minimum. By performing a treatment at 37°C for 60 minutes on 20 μl of a solution [50 mM NaCl, 100 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 8 units EcoR I (Nippon Gene Co.)] containing 1 μg pUC 19 DNA, a straight-chain vector DNA was obtained. This reaction solution was treated at 90°C for 5 minutes to deactivate the restriction enzyme, after which 38 μl H_2O and 1 unit bacterial alkaline phosphatase (Takara Shuzo Co.) were added to make a total of 60 μl, and a treatment was performed at 37°C for 60 minutes. This solution was treated with phenol and the water phase obtained was submitted to ethanol precipitation. The ethanol precipitate was freeze-dried and used in the following reaction.

The dephosphorylated pUC 19 vector (30 ng) and the phosphorylated double-chain DNA encoding the signal peptide

(10 ng) were treated at 15°C for 4 hours in a total of 30 μl of a reaction solution [66 mM Tris-HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP] containing 2.8 units of T4 DNA ligase (Takara Shuzo Co.), and a recombinant plasmid was obtained. Ten μl of this reaction solution were used for transforming the characteristics of the host bacterium, the coliform bacterium TB-1 strain.

The sensitive coliform bacteria cells used in the characteristic transformation can be prepared by, for example, the calcium chloride method [Mandel, M. and Higa, A., J. Mol. Biol. 53, 159-162 (1970)]. Specifically, an overnight culture solution of coliform bacteria (e.g., the TB-1 strain) [in an agar-agar medium, e.g., Luria (LB) medium] was diluted 100-fold with the same medium, and culturing with agitation was performed at 37°C until the OD 600 became 0.6. 1.5 μl were centrifuged at 5000 rpm for 5 minutes, and the bacteria were collected. These were suspended in 750 μl of 50 mM $CaCl_2$, and after leaving this on ice for 20 minutes, the bacteria were collected by centrifuging. The precipitate obtained was resuspended in 100 μl of 50 mM $CaCl_2$, and the aforementioned DNA ligase reaction solution was added; the resulting material (25 μg/ml) was left on ice for 40 minutes. After the temperature was held at 42°C for 1 minute, 1 ml LB medium was added and the temperature was held at 37°C for 30 minutes. 0.1 ml of

the result was applied to an X-Gal agar-agar medium (155 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 10 g tryptone, 8 g NaCl, and 12 g Difco agar-agar dissolved in 1 l water; pH adjusted to 7.4) containing ampicillin, and the temperature was held at 37°C overnight. From the colonies produced, the colonies which showed a white color were selected, transferred to a new agar-agar medium, and the temperature was held overnight. The bacteria were taken from this agar-agar medium with a platinum spatula, transferred to an LB medium, and an overnight cultured liquid was prepared. 1.5 ml of this overnight cultured liquid were centrifuged and the bacteria were collected; the mini-preparation of the plasmid DNA was performed by the usual method (Maniatis et al., Molecular Cloning: A Laboratory Manual, 1982). The plasmid DNA obtained was cut with a suitable restriction enzyme (e.g., one which cuts the recognition sequence contained in the synthetic DNA sequence inserted, such as EcoR I, Nae I, Hpa II, etc., or one which cuts the recognition sequence present in the pUC 19 vector, e.g., Pvu I, Bgl I, Ssp I, etc., or a combination of these). The length of the inserted DNA was checked by agarose and polyacrylamide gel electrophoresis, and the recombinant plasmid containing the suitable inserted DNA was identified. The DNA plasmid containing this inserted DNA was again put into M13mp phase DNA, the nucleotide sequence was determined by the dideoxy method [Sanger, F., Nicklen, S. and Corlson, A. R., Proc. Natl. Acad. Sci. U.S.A., 74, 5463-1564

(1977)], and finally, the target pUC-phoA plasmid was identified.

Reference Example 3. Preparation of plasmid pUC-phoA-HSA-A (Figs. 7-1, 7-2)

The plasmid pUC-phoA-HSA-A, containing DNA which encodes a fused protein composed of the signal peptide of coliform bacterial alkaline phosphatase (phoA) and normal human serum albumin A, was prepared as follows.

A fragment produced from clone lambda gt11 (HSA-II), containing HSA cDNA obtained from a human liver cDNA library, by digestion by EcoR I and Xba I, was prepared; this fragment was joined with the larger of the fragments obtained by double digestion of the pUC19 plasmid by EcoR I and Xba I, using T4 DNA ligase, and the recombinant plasmid pUC-HSA-EX was constructed.

The smaller of the fragments produced from this plasmid by double digestion by Aha III and Sal I was prepared. This fragment encodes [the part] from the 12th Lys to the 356th Thr of the mature normal human serum albumin A protein. In order to construct the genes which encode the mature normal human serum albumin A protein from the amine end, the DNA sequence corresponding to the 5′ end was made by annealing 2 chemically-synthesized fragments. This synthetic DNA sequence has the adhesion end sequence CG produced by cutting with the Hpa II and Cla I enzymes on the 5′ end side, so that it can fuse with the DNA sequence which

encodes the signal peptide of alkaline phosphatase, and it has the sequence which encodes [the part] from the first amino acid Asp to the 11th amino acid Phe of mature normal human serum albumin A. T4 polynucleotide kinase was caused to act on this annealed DNA sequence to phosphorylate the 5' end, and this was mixed with the product of double digestion by Aha III/Sal I, produced from pUC-HSA-EX. Furthermore, this was mixed with the larger of the fragments produced by double digestion by Cla I/Sal I of pAT153 (made by Amersham Co.; Twigg, A. J. and Sherratt, D., Nature 283, 216-218, 1980), a typical multi-copy cloning vector of coliform bacteria; these 3 [fragments] were joined by T4 DNA ligase, and the recombinant plasmid pAT-HSA-CX was obtained. On this plasmid, the DNA sequence encoding [the part] of the normal human serum albumin A from the first amino acid Asp to the 11th amino acid Phe was connected. The pAT-HSA-CX was double-digested by EcoR I/Xba I, and the smaller fragment, containing the DNA sequence which encodes [the part] of the normal human serum albumin A from Asp1 to Phe356 was obtained.

On the other hand, as for the cDNA which encodes the carboxyl end side of the HSA-A, an EcoR I fragment [into which a] foreign cDNA sequence from the clone lambda gt11 (HSA I-A), obtained from the human liver cDNA library, was inserted was prepared, and it was cloned in the recombinant plasmid pUC-HSA-1" by inserting [it] at the EcoR I site of

the pUC18 plasmid. In this way, [the part] of HSA-A from the 358th amino acid Leu to the 585th amino acid Leu of the carboxyl end was encoded; furthermore, a double digestion product by Xba I/Hind III, containing 62 nucleotides of the non-translation region of the 3′ side, was prepared. This was mixed with the larger of the fragments of the double digestion product of EcoR I/Xba I obtained from pAT-HSA-CX and the double digestion product of EcoR I/Hind III of pUC18; a linking reaction was performed by T4 DNA ligase, and the recombinant plasmid pUC-HSA-CH, containing all of the cDNA of the mature normal human serum albumin A, was obtained.

Figs. 8-1 to 8-3 show the cDNA base sequences which encode all the amino acid sequences of mature normal human serum albumin A and the corresponding amino acid sequences.

In order to join the cDNA of the mature normal human serum albumin A with the DNA sequence encoding the phoA signal peptide, the pUC-HSA-CH was cut with EcoR I/Cla I and the larger of the fragments produced was obtained. Using T4 DNA ligase, this was joined with the smaller of the fragments obtained by the double digestion of pUC-phoA by EcoR I/Msp I (cutting the same recognition sequence as Hpa II). The plasmid pUC-phoA-HSA-A constructed in this way contained a DNA sequence encoding a fused protein consisting of phoA signal peptide (consisting of 21 amino acids) and mature normal human serum albumin A; it was inserted in

coliform bacterium HB101 strain and cloned by the normal characteristic transformation method.

Reference Example 4. Preparation of plasmid pAT-trp-phoA-HSA-A

The expression plasmid pAT-phoA-HSA-A of normal human serum albumin A was constructed as follows. Using a vector containing trp promoter and the SD sequence of trpL, the expression vector of phoA-HSA-AcDNA was prepared. Such a vector is, for example, ph-TNF (Ikehara et al., Chem. Pharm. Bulletin, in press). This has the trp promoter and the SD sequence of trpL introduced into the pBR322 vector. When the number of copies of the recombinant plasmid is increased and a gene quantity effect is expected, a recombinant plasmid may be used which has for its basis pAT153 [Amersham; Twigg, A. J. and Sherratt, D. Nature, 283, 216-218 (1980)], which was prepared by removing the copy prevention sequence of pBR322. For example, this purpose can be accomplished by fusing the Pst I/Cla I double digestion product which contains the trp promoter/trpL SD sequence on ph-TNF with the larger of the fragments produced by the double digestion of pAT153 by the same combination of enzymes. The pAT-trp vector produced in this way was cut at the Cla I recognition site, one place downstream from the SD sequence, and the single-chain part of the adhesion end produced was buried by causing coliform bacteria DNA polymerase I to act on it; the straight-chain DNA produced was digested with Sal I. The

larger of the fragments obtained was used in the junction with phoA-HSA-AcDNA.

On the other hand, the smaller of the fragments produced by the double digestion of pUC-phoA-HSA-A by EcoR I/Hind III (containing the phoA-HSA-AcDNA sequence) was joined with the larger of the fragments produced by double digestion of pAT153 with EcoR I/Hind III, obtaining the recombinant plasmid pAT-phoA-HSA. After this was digested with EcoR I, making a straight-chain DNA, it was acted on by coliform bacteria DNA polymerase I to bury the single-chain part of the end. After this, it was cut with Sal I and the smaller of the fragments was recovered as the part containing the phoA-HSA-A cDNA. This fragment was joined with the previously-mentioned fragment from the pAT-trp vector, obtaining the recombinant plasmid pAT-trp-phoA-HSA-A.

This recombinant plasmid was introduced into the coliform bacteria strains HB101 and C600, and the characteristic transformation products E. coli HB101 (pAT-trp-phoA-HSA-A) and C600 (pAT-trp-phoA-HSA-A) were obtained.

The coliform bacterium C600 (pAT-trp-phoA-HSA-A), containing the recombinant plasmid pAT-trp-phoA-HSA-A which contains cDNA encoding the normal human serum albumin A, of this invention, was entrusted to the Microbiology Industry Technology Institute of the Agency of Industrial Science and Technology, as Bikokenkinki No. 9874 (FERM P-9874).

4. Simple Explanation of Figures:

Fig. 1 shows the base sequence of the synthetic DNA encoding [the sequence] from Met(123) to Ala(151) in the DNA encoding the human serum albumin fragment of this invention, and the corresponding amino acid sequence.

Fig. 2 shows the process of making the plasmids pUC-HSA-I and pSAL II from the cDNA clone lambda gt11 (HSA-I).

Fig. 3 shows the process of making the expression plasmid pAT-trp-phoA-SAL II of this invention.

Fig. 4 is an electrophoresis diagram of the expression product from the plasmid pAT-trp-phoA-SAL II; it shows a protein which reacted with the anti-human serum albumin antibodies.

Fig. 5 shows the base sequences of 3 probes used in screening the cDNA.

Fig. 6 shows the cDNA (HSA cDNA) which encodes all of the normal human serum albumin A, as the starting material of the plasmid of this invention, and a limiting enzyme map of the cDNA (HSA-IA) which encodes the 3' end side and the cDNA (HSA-II) which encodes the 5' end side, used to produce this cDNA.

Figs. 7-1 and 7-2 show the processes of producing various intermediate plasmids for producing the plasmid of this invention.

Figs. 8-1 to 8-3 show base sequences of the cDNA encoding all of the normal human serum albumin A of this invention. In the figures, the sequence within [ ], from amino acid 152 to amino acid 303, shows the amino acid sequence of the C-end side of the human serum albumin protein fragment of this invention and the base sequence encoding it.

Fig. 9 shows the process of producing the plasmids pUC-phoA-mHSA and pAT-trp-phoA-mHSA.

Fig. 10 shows the process of producing the plasmids pUC-tHSA and pAT-trp-tHSA.

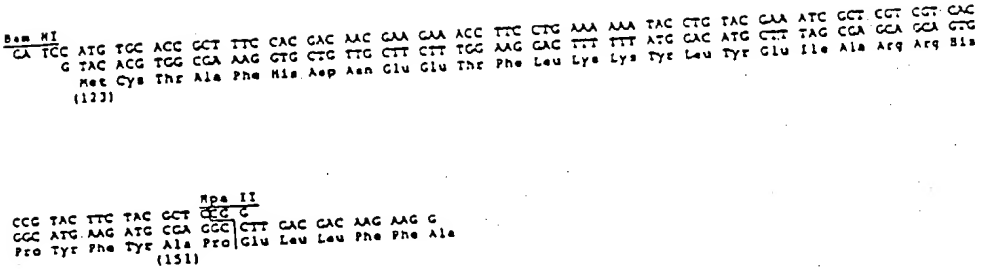
Fig. 11 shows the process of producing the plasmid pAT-trp-phoA-tHSA.

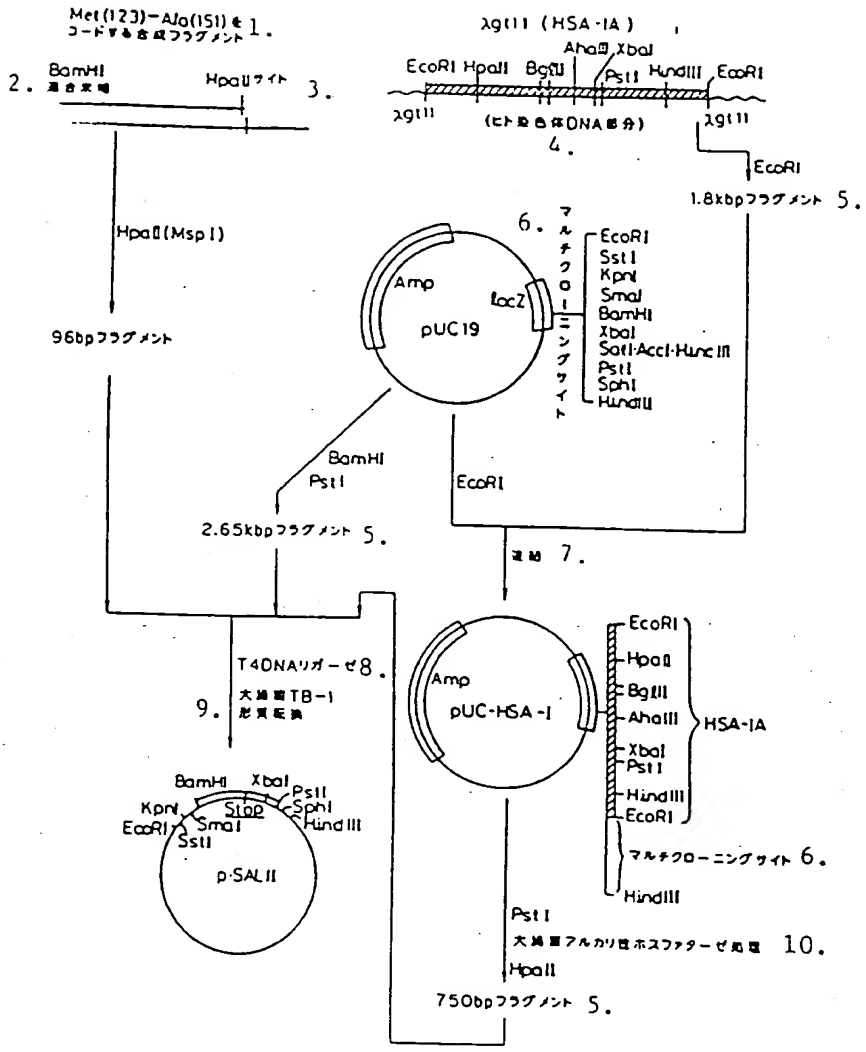
Fig. 12 is an SDS-polyacrylamide gel electrophoresis diagram of the expression products of the plasmid pAT-trp-phoA-mHSA (lane 4), pAT-trp-tHSA (lane 2), and pAT-trp-phoA-tHSA (lane 3); the protein bands were stained with Coomasie Brilliant Blue. Lane 1 represents the size markers: phosphorylase B (molecular weight 94,000), bovine serum albumin (molecular weight 67,000), ovalbumin (molecular weight 43,000), carboxylic acid dehydrogenase (molecular weight 30,000), soybean trypsin inhibitor (molecular weight 20,000), and lactoalbumin (molecular weight 14,400). The arrows indicate the various expression products.

Fig. 13 is a Western blot diagram of the expression products from pAT-trp-mHSA (lane 1), pAT-trp-tHSA (lane 3), and pAT-trp-phoA-tHSA (lane 2); it shows the proteins which reacted with the anti-human serum albumin antibodies.

Fig. 1

Fig. 1

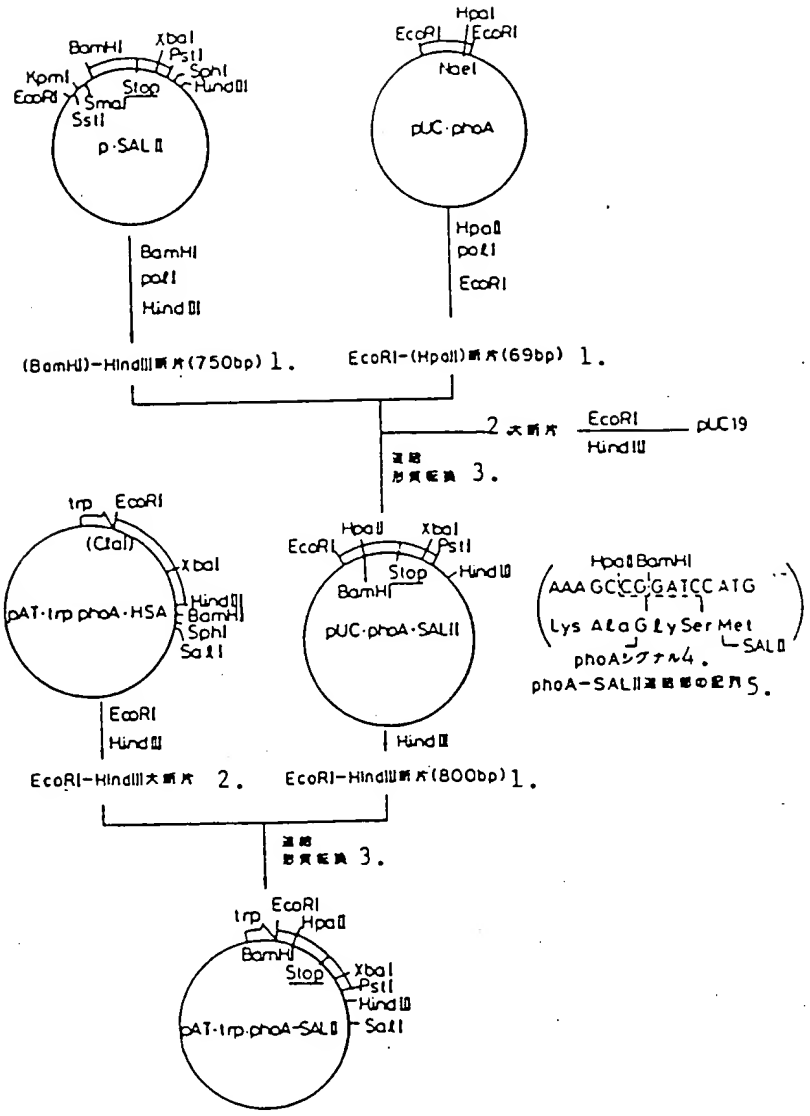

Bam HI
GA TCC ATG TGC ACC GCT TTC CAC GAC AAC GAA GAA ACC TTC CTG AAA AAA TAC CTG TAC GAA ATC GCT CGT CGT GAC
G TAC ACG TGG CGA AAG GTG CTG TTG CTT CTT TGG AAG GAC TTT TTT ATG GAC ATG CTT TAG CGA GCA GCA CTG
Met Cys Thr Ala Phe His Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg Arg His
(123)
Mpa II
CCG TAC TTC TAC GCT CCC G
GGC ATG AAG ATC CGA GGC CTT GAC GAC AAG AAG G
Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala
(151)



Key to Fig. 2

1. Synthetic fragment encoding Met(123)-Ala(151)
2. Junction end
3. Site
4. (Human chromosomal DNA part)
5. Fragment
6. Multi-cloning site
7. Joining
8. Ligase
9. Coliform bacteria TB-1 characteristic transformation
10. Coliform bacteria alkaline phosphatase treatment

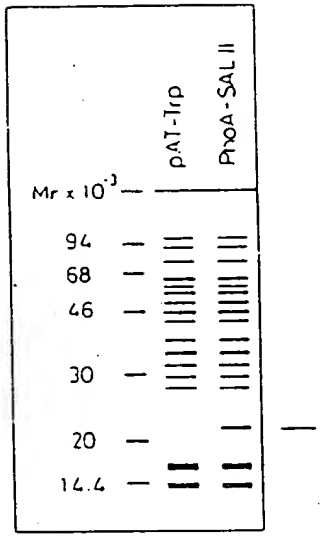
Fig. 3



Key to Fig. 3

1. Fragment
2. Large fragment
3. Joining, characteristic transformation
4. Signal
5. Sequence of phoA-SAL II junction part

Fig. 4



HSA -1 5′-AAGGGAAATAAAGGTTACCCACTTCATTGTGCCAAAGGC-3′

1. 5′-非翻訳領域-Met1-Leu9に相当する領域
2. (12ヌクレオチド)

HSA -2 5′-AAGGTCCGCCCTGTCATCAGCACATTCAAGCAGATCTCC-3′

Gly248-Leu260に相当する領域 3.

HSA -3 5′-TAGATGTTATAAGCCTAAGGCAGCTTGACTTGCAGCAAC-3′

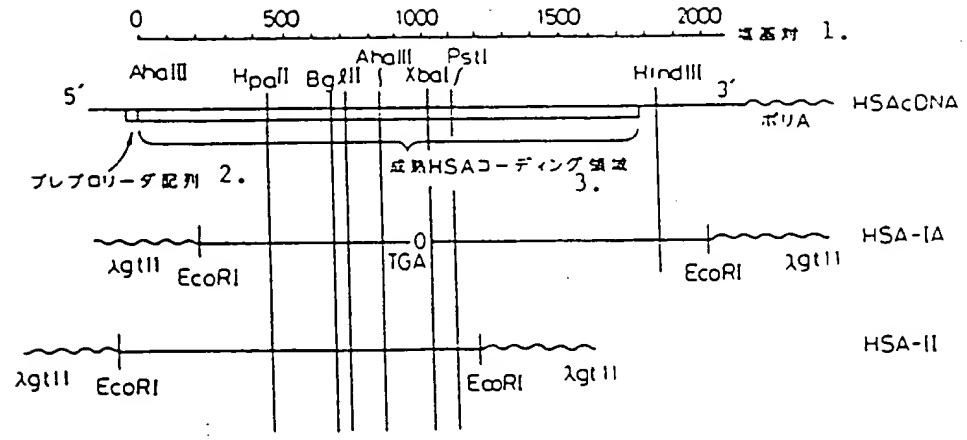
Val576-Leu585-3′非翻訳領域に相当する領域 4.
(6ヌクレオチド) 5.

Fig. 5

Key to Fig. 5

1. Region corresponding to 5′-non-translation region-Met1-Leu9
2. (12 nucleotides)
3. Region corresponding to Gly248-Leu260
4. Region corresponding to Val576-Leu585-3′ non-translation region
5. (6 nucleotides)

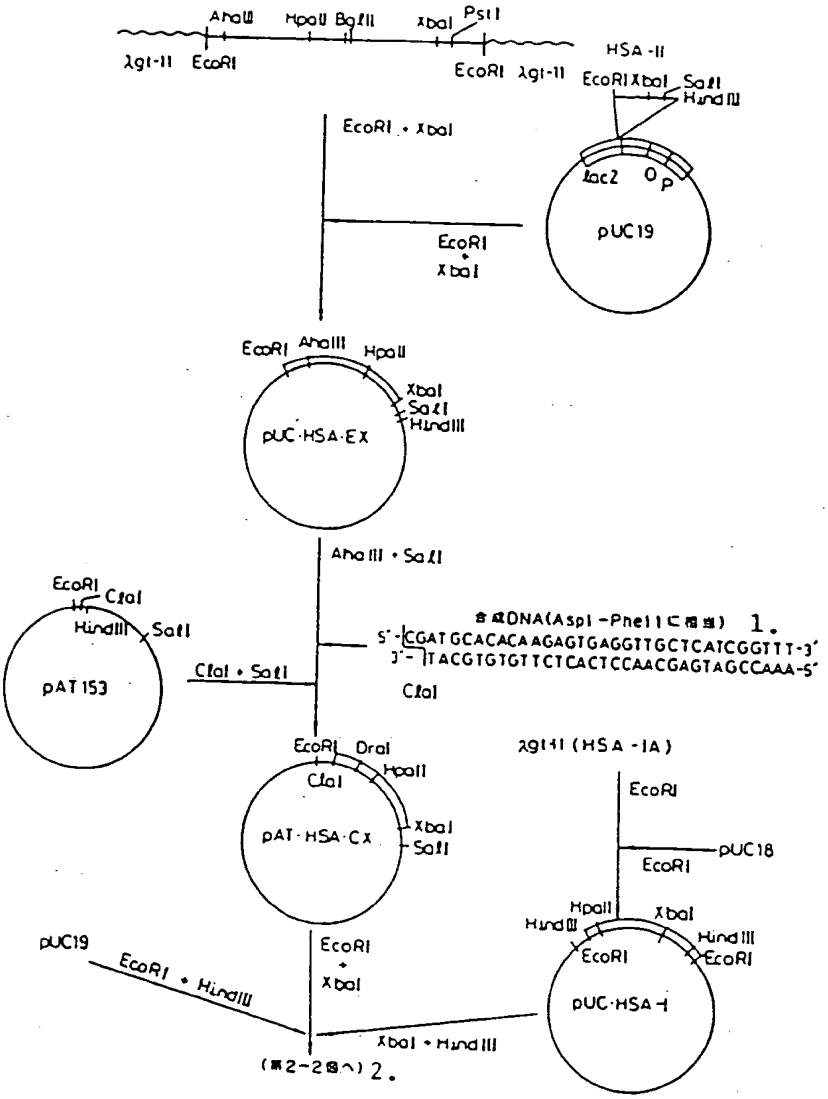
Fig. 6



Key to Fig. 6

1. Base pairs
2. Prepro leader sequence
3. Mature HSA coding region

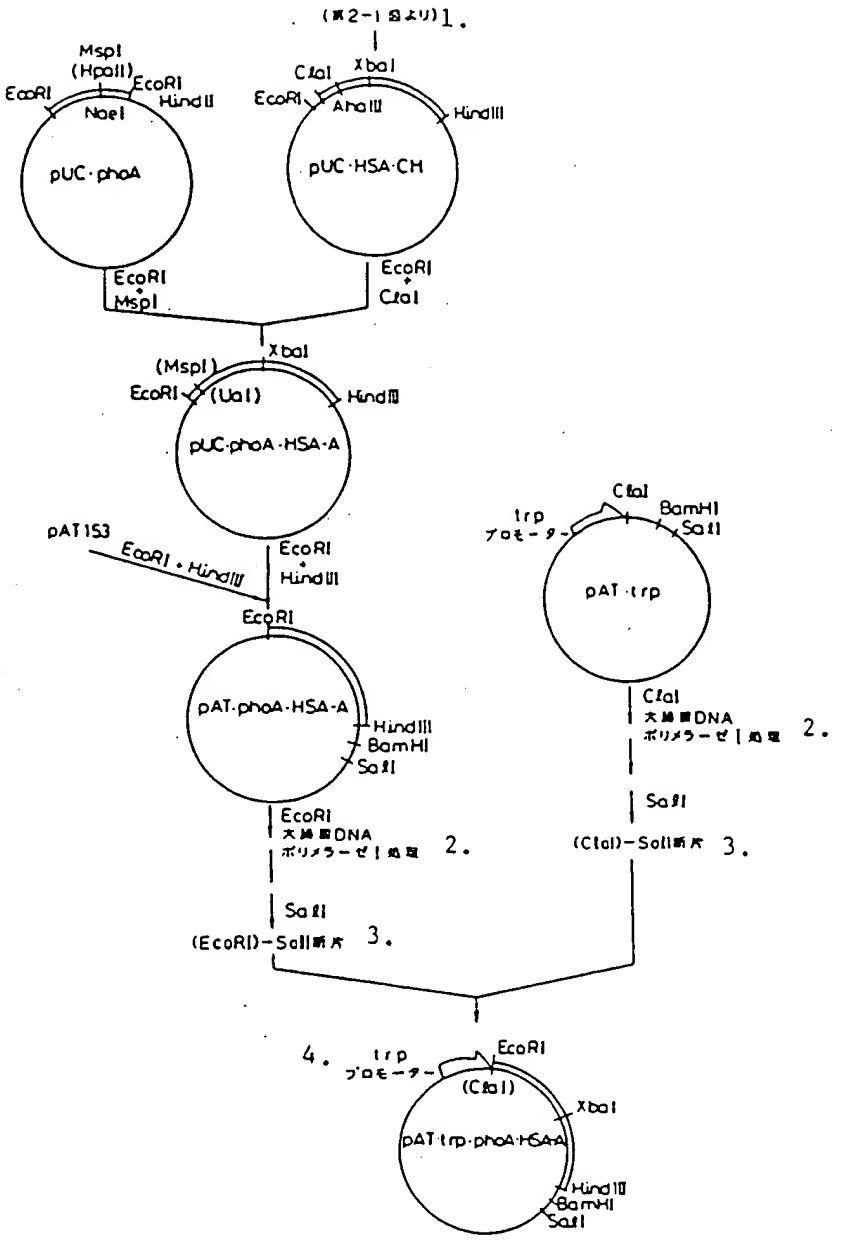
Fig. 7-1



Key to Fig. 7-1

1. Synthetic DNA (corresponding to Asp1-Phe11) .
2. (To Fig. 2-2) [apparently misprint for "7-2"-Trans_]

Fig. 7-2



Key to Fig. 7-2

1. (From Fig. 2-1) [apparently misprint for "7-1"-Trans.]
2. Coliform bacteria DNA polymerase I treatment
3. Fragment
4. Promoter

```
Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys Asp Leu Gly Glu Glu asn Phe Lys Ala Leu Val Leu Ile
GAT GCA CAC AAG AGT GAG GTT GCT CAT CGG TTT AAA GAT TTG GGA GAA GAA AAT TTC AAA GCC TTG GTG TTG ATT

                                                                                                  50
Ala Phe Ala Gln Tyr Leu Gln Gln Cys Pro Phe Glu Asp His Val Lys Leu Val Asn Glu Val Thr Glu Phe Ala
GCC TTT GCT CAG TAT CTT CAG CAG TGT CCA TTT GAA GAT CAT GTA AAA TTA GTG AAT GAA GTA ACT GAA TTT GCA

Lys Thr Cys Val Ala Asp Glu Ser Ala Glu Asn Cys Asp Lys Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys
AAA ACA TGT GTT GCT GAT GAG TCA GCT GAA AAT TGT GAC AAA TCA CTT CAT ACC CTT TTT GGA GAC AAA TTA TGC

                                                                                                 100
Thr Val Ala Thr Leu Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro Glu Arg Asn Glu
ACA GTT GCA ACT CTT CGT GAA ACC TAT GGT GAA ATG GCT GAC TGC TGT GCA AAA CAA GAA CCT GAG AGA AAT GAA

Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu Pro Arg Leu Val Arg Pro Glu Val Asp Val Met Cys Thr
TGC TTC TTG CAA CAC AAA GAT GAC AAC CCA AAC CTC CCC CGA TTG GTG AGA CCA GAG GTT GAT GTG ATG TGC ACT

                                                                                                 150
Ala Phe His Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg Arg His Pro Tyr Phe Tyr
GCT TTT CAT GAC AAT GAA GAG ACA TTT TTG AAA AAA TAC TTA TAT GAA ATT GCC AGA AGA CAT CCT TAC TTT TAT

Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala
GCC CCG GAA CTC CTT TTC TTT GCT AAA AGG TAT AAA GCT GCT TTT ACA GAA TGT TGC CAA GCT GCT GAT AAA GCT

                                                                                                 200
Ala Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser Ser Ala Lys Gln Arg Leu Lys Cys
GCC TGC CTG TTG CCA AAG CTC GAT GAA CTT CGG GAT GAA GGG AAG GCT TCG TCT GCC AAA CAG AGA CTC AAG TGT
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8-2

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Ala Ser Leu Gln Lys Phe Gly Glu Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Arg Phe Pro Lys
GCC AGT CTC CAA AAA TTT GGA GAA AGA GCT TTC AAA GCA TGG GCA GTA GCT CGC CTG AGC CAG AGA TTT CCC AAA

                                                                                                    250
Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys Val His Thr Glu Cys Cys His Gly Asp Leu
GCT GAG TTT GCA GAA GTT TCC AAG TTA GTG ACA GAT CTT ACC AAA GTC CAC ACG GAA TGC TGC CAT GGA GAT CTG

Leu Glu Cys Ala Asp Asp Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser Ser Lys Leu
CTT GAA TGT GCT GAT GAC AGG GCG GAC CTT GCC AAG TAT ATC TGT GAA AAT CAA GAT TCG ATC TCC AGT AAA CTG

                                                                                                    300
Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala
AAG GAA TGC TGT GAA AAA CCT CTG TTG GAA AAA TCC CAC TGC ATT GCC GAA GTG GAA AAT GAT GAG ATG CCT GCT

Asp Leu Pro Ser Leu Ala Ala Asp Phe Val Glu Ser Lys Asp Val Cys Lys Asn Tyr Ala Glu Ala Lys Asp Val
GAC TTG CCT TCA TTA GCT GCT GAT TTT GTT GAA AGT AAG GAT GTT TGC AAA AAC TAT GCT GAG GCA AAG GAT GTC

                                                                                                    350
Phe Leu Gly Met Phe Leu Tyr Glu Tyr Ala Arg Arg His Pro Asp Tyr Ser Val Val Leu Leu Leu Arg Leu Ala
TTC CTG GGC ATG TTT TTG TAT GAA TAT GCA AGA AGG CAT CCT GAT TAC TCT GTC GTG CTG CTG CTG AGA CTT GCC

Lys Thr Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala Ala Ala Asp Pro His Glu Cys Tyr Ala Lys Val Phe Asp
AAG ACA TAT GAA ACC ACT CTA GAG AAG TGC TGT GCC GCT GCA GAT CCT CAT GAA TGC TAT GCC AAA GTG TTC GAT

                                                                                                    400
Glu Phe Lys Pro Leu Val Glu Glu Pro Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu
GAA TTT AAA CCT CTT GTG GAA GAG CCT CAG AAT TTA ATC AAA CAA AAT TGT GAG CTT TTT GAG CAG CTT GGA GAG
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8-3

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Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro Gln Val Ser Thr Pro Thr Leu Val Glu
TAC AAA TTC CAG AAT GCG CTA TTA GTT CGT TAC ACC AAG AAA GTA CCC CAA GTG TCA ACT CCA ACT CTT GTA GAG

                                                                                                    450
Val Ser Arg Asn Leu Gly Lys Val Gly Ser Lys Cys Cys Lys His Pro Glu Ala Lys Arg Met Pro Cys Ala Glu
GTC TCA AGA AAC CTA GGA AAA GTG GGC AGC AAA TGT TGT AAA CAT CCT GAA GCA AAA AGA ATG CCC TGT GCA GAA

Asp Tyr Leu Ser Val Val Leu Asn Gln Leu Cys Val Leu His Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys
GAC TAT CTA TCC GTG GTC CTG AAC CAG TTA TGT GTG TTG CAT GAG AAA ACG CCA GTA AGT GAC AGA GTC ACA AAA

                                                                                                    500
Cys Cys Thr Glu Ser Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr Tyr Val Pro Lys
TGC TGC ACA GAG TCC TTG GTG AAC AGG CGA CCA TGC TTT TCA GCT CTG GAA GTC GAT GAA ACA TAC GTT CCC AAA

Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp Ile Cys Thr Leu Ser Glu Lys Glu Arg Gln Ile Lys Lys
GAG TTT AAT GCT GAA ACA TTC ACC TTC CAT GCA GAT ATA TGC ACA CTT TCT GAG AAG GAG AGA CAA ATC AAG AAA

                                                                                                    550
Gln Thr Ala Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu Lys Ala Val Met Asp Asp
CAA ACT GCA CTT GTT GAG CTT GTG AAA CAC AAG CCC AAG GCA ACA AAA GAG CAA CTG AAA GCT GTT ATG GAT GAT

Phe Ala Ala Phe Val Glu Lys Cys Cys Lys Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu
TTC GCA GCT TTT GTA GAG AAG TGC TGC AAG GCT GAC GAT AAG GAG ACC TGC TTT GCC GAG GAG GGT AAA AAA CTT

Val Ala Ala Ser Gln Ala Ala Leu Gly Leu End
GTT GCT GCA AGT CAA GCT GCC TTA GGC TTA TAA
```
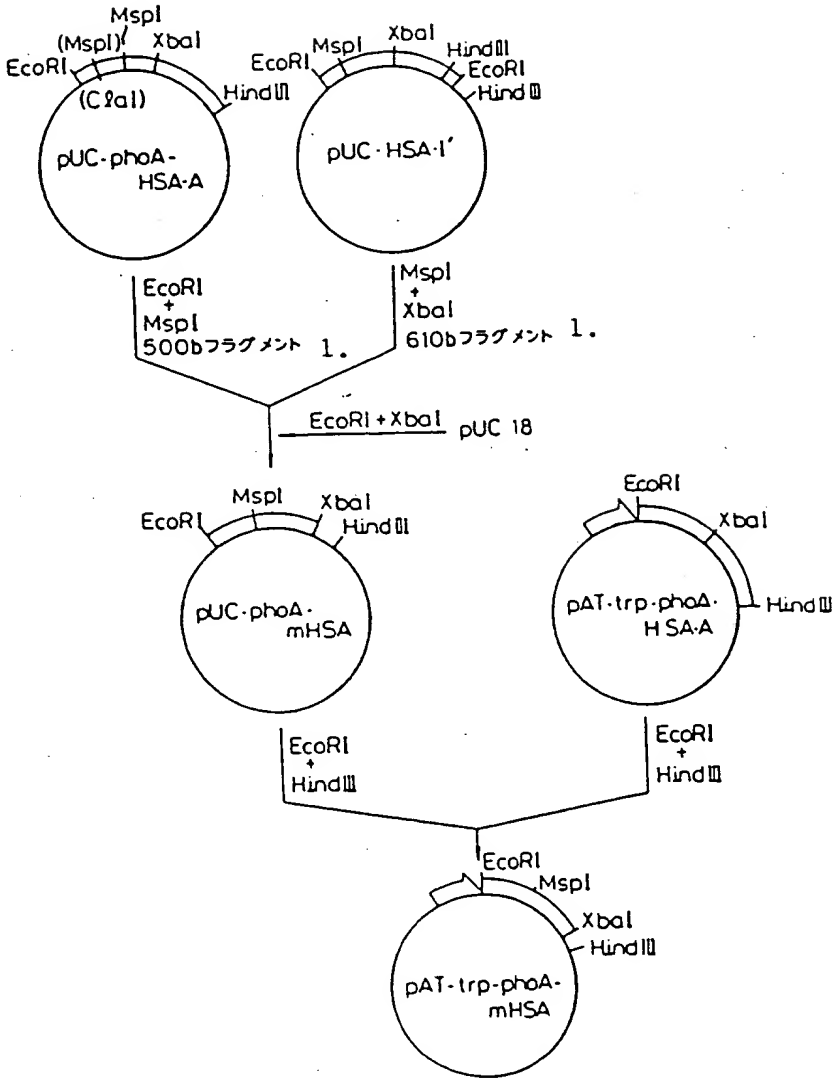
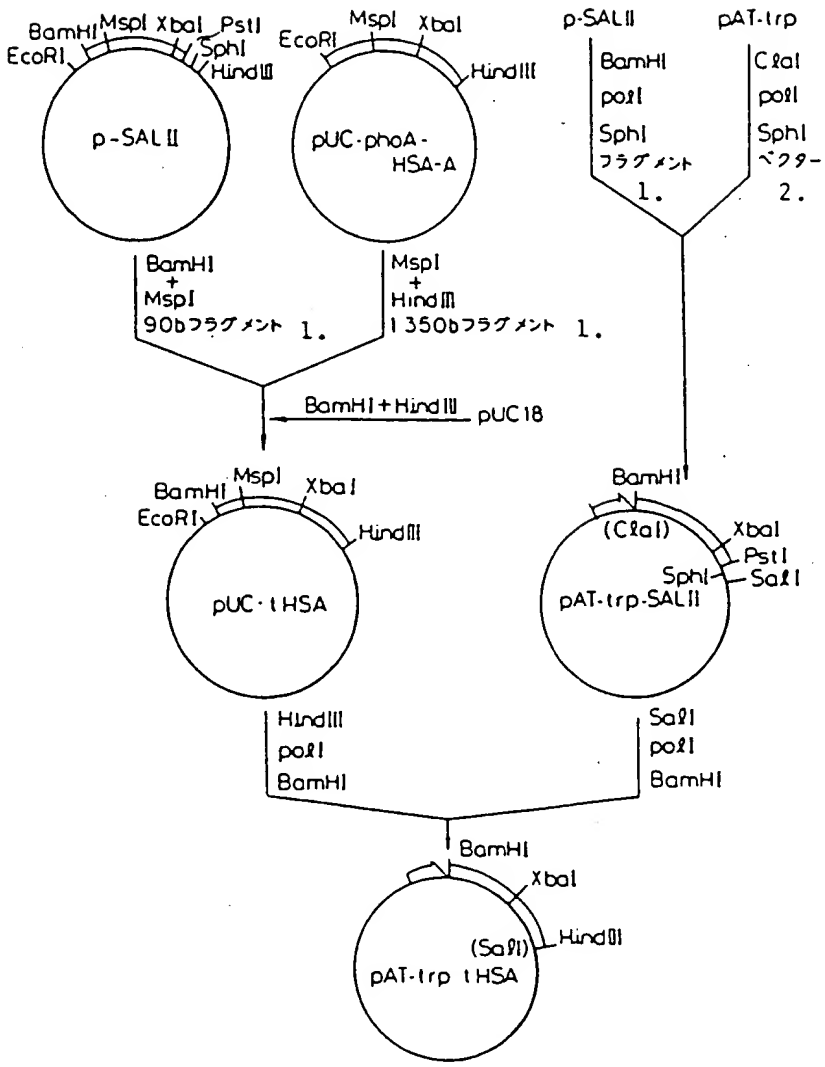

Fig. 9



Key to Fig. 9

1. Fragment

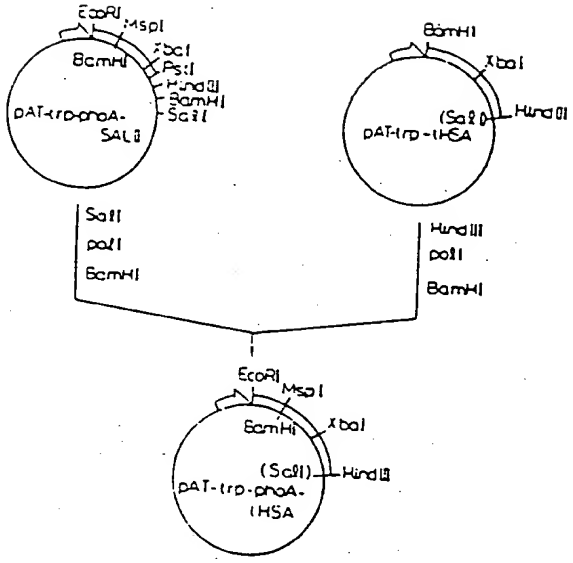
Fig. 10

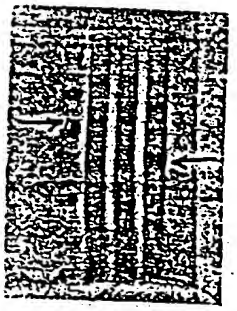


Key to Fig. 10

1. Fragment
2. Vector

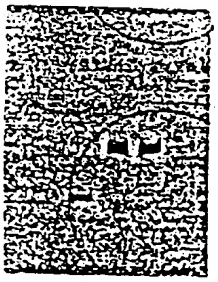
Fig. 11





1 2 3 4

Fig. 12



1 2 3

Fig. 13